

## Influence of Involution on Intramammary Phagocytic Defense Mechanisms<sup>1,2</sup>

M. J. PAAPE and R. H. MILLER  
Milk Secretion and Mastitis Laboratory  
Agricultural Research Service, USDA  
Beltsville, MD 20705-2350

M. D. YOUNG and R. R. PETERS  
Department of Animal Sciences  
University of Maryland  
College Park 20742-2311

### ABSTRACT

Mammary secretions (n = 34 cows) and mammary phagocytes (n = 18 cows) were collected throughout the nonlactating (dry) period to determine changes in intramammary phagocytic defense mechanisms. Mammary secretions were evaluated for their ability to support phagocytosis of *Staphylococcus aureus* by neutrophils from donor cows and mammary phagocytes for phagocytic and chemiluminescence activity.

Ability of secretions to support phagocytosis decreased with advancing length of the dry period. This effect was more pronounced when dry cow secretions constituted 50% of the phagocytic mixture. Phagocytic activity of mammary phagocytes decreased with advancing dry period when autologous secretion was used in the incubation mixture. With homologous secretion, the percentage of phagocytosis increased 5 to 6 d after drying off compared with before drying off and then gradually decreased throughout the remainder of the dry period. Chemiluminescence activity (log<sub>10</sub> counts per minute) of mammary phagocytes was lower during the dry period and decreased with advancing dry period.

Results indicated diminishing ability of secretions to support phagocytosis and diminished phagocytic and bactericidal mechanisms during the dry period.

(Key words: chemiluminescence, mammary gland, phagocytosis)

Abbreviation key: CL = chemiluminescence.

### INTRODUCTION

The incidence of new intramammary infection is greatest during the first 2 wk of the dry period and during the first 2 wk after parturition (8, 28). The simultaneous discoveries by Guidry et al. (12) and Newbould (29), that the decrease in phagocytic activity of circulating neutrophils during the first 2 wk after parturition could contribute to increased susceptibility to infection, prompted several studies on phagocyte function during the dry and immediate postpartum periods. Consistent with this finding was the decrease in the in vitro chemotaxis by neutrophils isolated from blood on the day of and immediately after calving (4, 19, 26). The ability of circulating neutrophils to phagocytose during this time was either depressed (5, 33) or unchanged (4, 19). Paradoxical results have also been reported on the ability of mammary phagocytes to phagocytose. Two studies reported decreased phagocytosis of *Staphylococcus aureus* during the late dry period (40 to 45 d) (3, 16), and one reported an increase (36). During the early dry period, increased phagocytosis of *S. aureus* and decreased phagocytosis of *Escherichia coli* was observed on d 14 compared with that on d 7 (9). Another study using *Streptococcus dysgalactiae* reported increased phagocytosis on d 12 compared with d 9 (14). Although the strain of organism used could have contributed to

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these varying results, other possible factors could be differences in the source and concentration of opsonins used and differences in the way that the phagocytosis assays were conducted. Because of the differing results obtained on the ability of mammary phagocytes to phagocytose, an investigation into effects of differing concentrations of autologous and homologous mammary secretions on phagocytosis appeared to be justified.

There are no studies comparing changes in mammary phagocyte bactericidal activity before and throughout the dry period. One study (9) compared d 7 with 14 of the dry period and reported no change in intracellular killing of bacteria by mammary phagocytes. Two studies (19, 26) compared changes in microbicidal mechanisms of circulating neutrophils around parturition and reported an increase 2 wk before parturition followed by a decrease during the 1st wk after parturition. An examination of mammary phagocyte bactericidal mechanisms during lactation and mammary involution also appeared to be crucial to further understanding of phagocytic defense during mammary involution.

Although a number of studies have been conducted on phagocytic activity of neutrophils isolated from blood and dry cow secretions, work has been limited on ability of mammary secretions collected throughout the dry period to support phagocytosis. Two studies reported that mammary secretions obtained at calving and at 7 d postpartum were inhibitory to phagocytosis (34, 36). An increase in the ability of mammary secretions to support phagocytosis at d 7 and 14 after drying off was reported by Miller et al. (23). Most new intramammary infections during the early dry period occur by d 7 after drying off (8), which we thought warranted a more intensive investigation throughout the first 7 d after drying off, and at various intervals throughout the dry period, on the ability of differing concentrations of mammary secretions to support phagocytosis. In a previous paper (24), we reported on the changes in total and differential somatic cells and N-acetyl- $\beta$ -D-glucosaminidase activity during those times.

The objectives of this study were 1) to study changes in phagocytosis and chemiluminescence (CL) activity of mammary phagocytes before and after drying off and 2) to

examine changes in the ability of mammary secretions—collected during the first 7 d after dry-off and at various times throughout the dry period—to support phagocytosis of neutrophils obtained from donor cows.

## MATERIALS AND METHODS

### Cattle

Thirty-four cows were used as sources of mammary secretions and 18 as sources of mammary leukocytes. On the basis of results of two consecutive daily bacteriological cultures (27) of mammary secretions on blood-esculin agar, these cattle were free of intramammary infection before the start of the experiment. Cows were not treated with antibiotics at drying off.

### Experiment 1: Ability of Secretions to Support Phagocytosis

Mammary secretions were collected from all four quarters of 16 cows at 7, 14, and 21 d after drying off. Aseptic samples were simultaneously obtained for bacteriological analysis (28).

### Experiment 2: Ability of Secretions to Support Phagocytosis and Phagocyte Function

Mammary secretions were collected from 18 cows, not used in Experiment 1, at intervals before drying off, at drying off, and d 2, 3, 4, 5, 7, between 8 to 14 d, between 15 to 22 d, and on the day of calving (23 to 65 d after drying off). Aseptic samples were simultaneously obtained for bacteriological analysis (28). Between 7 to 14 d before drying off, mammary gland neutrophils were isolated 14 h after intramammary injection of 50  $\mu$ g of *E. coli* endotoxin (Difco Laboratories, Detroit, MI). Mammary secretions were collected from uninjected mammary quarters on d 5 and 7, then on 11 to 14 d, and on 15 to 22 d after drying off. From 50 to 250 ml of secretion were collected (by hand milking) into a polypropylene flask.

### Mammary Secretions

Secretions were centrifuged at  $6500 \times g$  for 30 min at 4°C, and the cream layer was re-

moved. The sample was recentrifuged, and the residual cream was removed by aspiration. The skimmed secretion was decanted carefully to avoid disturbing the sediment. Secretions were centrifuged at  $46,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  to remove casein. Samples were kept frozen until assayed for their ability to support phagocytosis.

#### Phagocytes

Samples and reagents were kept at  $5^{\circ}\text{C}$  during the isolation procedure. Secretions were filtered through silk cloth (NSG Precision Cells Inc., Hicksville, NY). A portion was saved and skimmed as previously described for use in phagocytosis assays. The remainder was diluted to 50% with .01 M .85% PBS, poured into siliconized bottles (Corex, Du Pont Co., Newtown, CO), and centrifuged at  $1000 \times g$  for 15 min. The cream layer was removed, the skimmed secretion was discarded, and the cell pellet was gently suspended in 80 ml of PBS. After centrifugation at  $200 \times g$ , cells were washed twice in PBS. The sedimented cells were suspended in PBS solution, and the phagocytes (neutrophils and macrophages) were adjusted to desired concentrations. Because of the poor separation efficiency of the different leukocytes in involuted mammary secretions (18), no attempt was made to separate neutrophils from macrophages.

Cells were enumerated using an electronic cell counter (Coulter Electronics Inc., Hialeah, FL). Cell viability was determined by trypan blue dye (Allied Chemical, Morristown, NJ) exclusion, and differential cell counts were performed on Wright-stained (Miles Inc., Elkhart, IN) smears.

#### Phagocytosis

Percentage of phagocytosis was measured after 60 min of incubation as described (7). The ability of mammary secretions to support phagocytosis was determined by incubating mammary neutrophils, isolated (31) from two lactating donor cows, with  $^{32}\text{P}$ -labeled *S. aureus* in mammary secretions. The incubation mixture included 1 ml of undiluted or diluted secretions, .5 ml of the neutrophil suspension ( $1.25 \times 10^7$ ), and .5 ml of  $^{32}\text{P}$ -labeled *S. aureus* ( $2 \times 10^8$ ). All determinations were

performed in duplicate. The ability of phagocytes to phagocytose was determined by incubating .5 ml of the cell suspension ( $1.25 \times 10^7$ ) and .5 ml of  $^{32}\text{P}$ -labeled *S. aureus* ( $2 \times 10^8$ ) in 1 ml each of either 100 or 5% autologous secretion or in 1 ml of a 5% pooled homologous skim milk that was prepared from 4 donor cows (91 to 152 d of lactation) not used in the study.

#### CL

Luminol-enhanced CL was measured in a liquid scintillation counter (LS 100C, Beckman Instruments Inc., Silver Spring, MD) at  $21^{\circ}\text{C}$ , using the tritium channel and the coincidence mode as previously described (6). Briefly, isolated phagocytes ( $5 \times 10^6/2$  ml) were added to 7-ml scintillation vials containing luminol (1.8 ml) and zymosan ( $5 \times 10^8/\text{ml}$ ) or balanced salt solution (1 ml). Vials were counted for 1 min at 10-min intervals for a maximum of 100 min. All determinations were performed in duplicate.

#### Statistical Analysis

Trends during the dry period were analyzed by least squares analysis of variance. The model for percentage of phagocytosis included effects of run date, neutrophil donor cow, and cow sampled, effects of dilution, categories of stage of dry period, and interaction of dilution with stage of dry period.

The model for CL included effects of cow, quarter, and stage of dry period. For each stage of dry period, the CL response curve was estimated. Duplicate determinations were averaged. Counts per minute were transformed to  $\log_{10}$  for analysis. For the CL comparisons using zymosan, counts per minute in the absence of zymosan (balanced salt solution alone) were subtracted from counts per minute in the presence of zymosan during each period.

## RESULTS

#### Experiment 1: Ability of Secretions to Support Phagocytosis

During Experiment 1, 24 quarters became infected with either major or minor pathogens. Intramammary infection had no effect on the

TABLE 1. Effect of infection status on ability of secretions to support phagocytosis of  $^{32}\text{P}$ -labeled *Staphylococcus aureus* by bovine neutrophils (Experiment 1).

Days dry	Infected (n = 24 quarters)	Uninfected (n = 40 quarters)	Significance <sup>1</sup>
	— (% phagocytosed) —		
7	79 <sup>a</sup>	76 <sup>a</sup>	NS
14	74 <sup>b</sup>	70 <sup>b</sup>	NS
21	71 <sup>c</sup>	69 <sup>b</sup>	NS

<sup>a,b,c</sup>Means within the same column with no common superscript letters differ ( $P < .05$ ).

<sup>1</sup>Infected versus uninfected ( $P > .05$ ).

ability of secretions to support phagocytosis (Table 1). Although there was a tendency at all stages of the dry period for phagocytosis to be higher in infected quarters than in uninfected quarters, differences were not significant ( $P > .05$ ). The ability of secretions to support phagocytosis decreased ( $P < .05$ ) with stage of the dry period. The concentration of secretion in the incubation mixture significantly affected phagocytosis (Table 2). The ability of secretions to support phagocytosis was greater ( $P < .05$ ) on d 7 and 14 when secretions constituted 2.5% of the incubation mixture than when they constituted 25%. For both concentrations of secretions, significant ( $P < .05$ ) depressions in ability to support phagocytosis occurred with advancing length of the dry period.

#### Experiment 2: Ability of Secretions to Support Phagocytosis

Concentration of secretion in the incubation mixture had a significant effect on phagocytosis (Table 3). When the secretions constituted 50% of the incubation mixture, phagocytosis was greater ( $P < .05$ ) at 21 and 16 d before drying off, on the day of drying off, and at calving (23 to 65 d) compared with a secretion concentration of 2.5%. Conversely, phagocytosis was greater from d 1 to 22 of the dry period when the secretion concentration was 2.5%. Variation throughout the dry period in the ability of concentration of secretion to support phagocytosis contributed to a significant concentration by time interaction ( $P < .01$ ). Changes ( $P < .05$ ) in the ability of secretions to support phagocytosis occurred throughout the dry period (Table 3). Fluctuations in the

TABLE 2. Changes in the ability of dry cow secretions to support phagocytosis of  $^{32}\text{P}$ -labeled *Staphylococcus aureus* by bovine neutrophils (Experiment 1).

Days dry	Concentration of secretion in incubation mixture		Significance
	25%	2.5%	25 vs. 2.5%
	— (% phagocytosed) —		
7	75 <sup>a</sup>	79 <sup>a</sup>	$P < .05$
14	70 <sup>b</sup>	74 <sup>b</sup>	$P < .05$
21	68 <sup>b</sup>	71 <sup>c</sup>	NS

<sup>a,b,c</sup>Means within the same column with no common superscript letters differ ( $P < .05$ ).

percentage of phagocytosis were greatest when the concentration of secretion in the incubation mixture was 2.5%. At this concentration, percentage of phagocytosis averaged 54% at 21 d before drying off, increased ( $P < .05$ ) to 81% at 7 d before drying off, decreased ( $P < .05$ ) to 57% at drying off, gradually increased ( $P < .05$ ) to 75% at 5 d after drying off, and decreased ( $P < .05$ ) to 26% at calving. When secretions constituted 50% of the incubation mixture, percentage of phagocytosis after drying off was similar ( $P > .05$ ) but lower ( $P < .05$ ) than before drying off.

TABLE 3. Changes in the ability of dry cow secretions to support phagocytosis of  $^{32}\text{P}$ -labeled *Staphylococcus aureus* by neutrophils from donor cows (Experiment 2).

Days dry	Concentration of secretion in incubation mixture		Significance
	50%	2.5%	50 vs. 2.5%
	— (% phagocytosed) —		
-21	76 <sup>ab</sup>	54 <sup>a</sup>	$P < .05$
-16	78 <sup>ab</sup>	57 <sup>a</sup>	$P < .05$
-7	82 <sup>a</sup>	81 <sup>b</sup>	NS
0	75 <sup>bc</sup>	57 <sup>a</sup>	$P < .05$
1	64 <sup>c</sup>	68 <sup>c</sup>	$P < .05$
3	62 <sup>c</sup>	74 <sup>d</sup>	$P < .05$
4	64 <sup>c</sup>	74 <sup>d</sup>	$P < .05$
5	62 <sup>c</sup>	75 <sup>d</sup>	$P < .05$
7	65 <sup>c</sup>	72 <sup>d</sup>	$P < .05$
8 to 14	63 <sup>c</sup>	69 <sup>c</sup>	$P < .05$
15 to 22	63 <sup>c</sup>	68 <sup>c</sup>	$P < .05$
23 to 65 (Calving)	70 <sup>bc</sup>	26 <sup>e</sup>	$P < .05$

<sup>a,b,c,d,e</sup>Means within the same column with no common superscript letters differ ( $P < .05$ ).

TABLE 4. Changes in the ability of mammary phagocytes to phagocytose (Experiment 2).

Range of dry period	Concentration of secretion in incubation mixture			Dry period mean
	50% Autologous	2.5% Autologous	2.5% Homologous	
(d)	— (% phagocytosed) —			
-7 to -14	92 <sup>a,x</sup>	85 <sup>a,x</sup>	63 <sup>b,xy</sup>	80 <sup>x</sup>
5 to 6	85 <sup>a,x</sup>	94 <sup>a,x</sup>	82 <sup>a,x</sup>	87 <sup>x</sup>
7	74 <sup>ab,x</sup>	82 <sup>a,x</sup>	62 <sup>b,xy</sup>	73 <sup>x</sup>
8 to 14	80 <sup>a,x</sup>	84 <sup>a,x</sup>	63 <sup>b,xy</sup>	76 <sup>x</sup>
15 to 22	56 <sup>a,y</sup>	66 <sup>a,y</sup>	56 <sup>a,y</sup>	60 <sup>y</sup>
Concentration mean	73 <sup>a</sup>	81 <sup>a</sup>	59 <sup>b</sup>	

<sup>a,b</sup>Means within the same row with no common superscript letters differ ( $P < .05$ ).

<sup>x,y</sup>Means within the same column with no common superscript letters differ ( $P < .05$ ).

#### Experiment 2: Phagocytosis

Mammary phagocytes on averages tended to be more phagocytic 5 to 6 d after drying off ( $P < .10$ ) and less phagocytic 15 to 22 d after drying off ( $P < .05$ ) (Table 4). There was no difference ( $P > .05$ ) between the 50 and 2.5% concentrations of autologous secretions used as sources of opsonins, and both supported greater phagocytosis than the 2.5% homologous skimmed milk.

Neutrophils constituted the major cell type in the leukocyte preparations used in the phagocytosis assays (Table 5). The percentage of neutrophils in the cell preparations decreased with advancing dry period, and the percentages of macrophages and lymphocytes increased.

#### Experiment 2: CL

Background CL activity was highest ( $P < .05$ ) for phagocytes collected from secretions during the dry period compared with activity of phagocytes collected before drying off (Figure 1). Both the rate and peak CL activity were greater for phagocytes from secretions collected during the dry period (Figure 2). Activity was highest for phagocytes collected before drying off.

#### DISCUSSION

Ability of mammary secretions to support phagocytosis, ability of mammary phagocytes

TABLE 5. Percentage differential of leukocyte preparations used in phagocytosis assays (Experiment 2).

Days dry	Macrophages	Neutrophils	Lymphocytes
-7 to -14	2	95	3
5 to 7	8	84	8
8 to 14	17	68	15
15 to 22	20	59	22

to phagocytose, and CL activity decreased with advancing dry period. Most notable were the decreases in functional activity of mammary phagocytes harvested on d 21 of the dry period and the ability of secretions to support phagocytosis at calving when a secretion concentration of 50% was used as the source of opsonins in the phagocytosis assay.

That the source and concentration of secretion in the incubation mixture significantly affected bacterial ingestion raised a number of questions. Regarding the source of the secretion, autologous dry cow secretions were apparently more supportive of phagocytosis than homologous skimmed milk secretions. However, homologous secretions were also less supportive of phagocytosis during the lactation phase of the study. This indicated that the milk from donor cows simply had reduced ability to support phagocytosis. Variation exists among cows in the ability of milk to support phagocytosis (31) and is positively correlated with Ig concentration (1, 22). Interestingly, increased

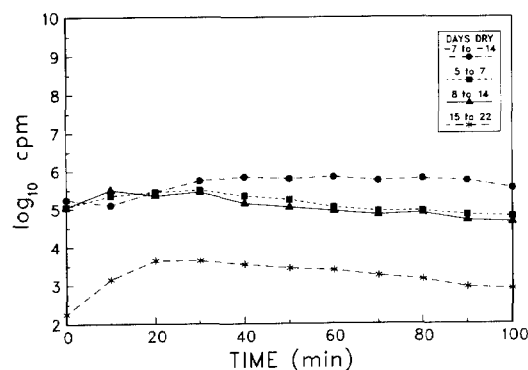


Figure 1. Chemiluminescence activity of mammary phagocytes in the absence of zymosan (background activity) relative to drying off (Experiment 2). Standard errors for before dry-off and 7, 14, and 21 d after drying off were .200, .156, .129, and .364, respectively.

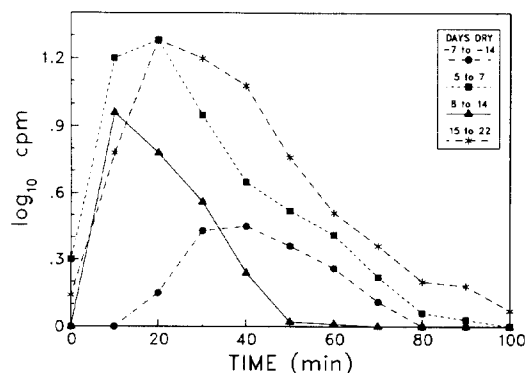


Figure 2. Chemiluminescence activity of mammary phagocytes in the presence of zymosan relative to drying off (Experiment 2). Standard errors for before dry-off and 7, 14, and 21 d after dry-off were .057, .044, .036, and .100, respectively.

concentration of secretion in the phagocytosis assay was more supportive of phagocytosis during certain phases of the study and inhibitory at other phases. During lactation and colostrogenesis, high secretion concentration was more supportive of phagocytosis. Throughout the involutionary and steady state phases of the dry period, high secretion concentrations were inhibitory. Inhibitory effects were greatest between d 5 and 7 of the dry period. Targowski and Niemialowski (36) reported that dry secretions collected on d 7 were more inhibitory to phagocytosis than those collected on d 42 of the dry period. The authors attributed this to a blockage of Fc receptors on the neutrophil surface by immune complexes. Mullan et al. (25) also reported that high concentrations of dry gland secretions were inhibitory to phagocytosis by mammary macrophages obtained at an unspecified time during the dry period.

At parturition, we attributed the large decrease in the ability of the 2.5% secretion concentration to support phagocytosis to the reported decline in the amounts of opsonic antibodies IgG<sub>2</sub> and IgM (4, 35). Why phagocytosis did not decrease at the 50% concentration of secretion could be attributable to several factors. The onset of colostrogenesis, while diluting Ig titers, would also dilute inhibitory effects of immune complexes on phagocytosis, resulting in no change in phagocytosis.

The depression in phagocyte function later in the dry period could be, in part, explained by the increased percentage of macrophages in the cell preparation. Macrophages have been reported (15, 25) to be less phagocytic than neutrophils.

There are no reports of CL activity of phagocytes obtained from mammary glands during the dry period. As a result of respiratory burst activation, neutrophils and macrophages produce highly unstable oxygen metabolites that are involved in the killing of bacteria (2). Chemiluminescence, the accompanying light emission, has been used to study oxygen-dependent microbicidal systems of phagocytes (17). The higher background CL activity of phagocytes harvested from secretions obtained during the dry period, compared with when they were harvested from milk, may be due to cell activation caused by increased concentrations of Ig, complement components, and immune complexes (13) reported to occur in secretions from involuted mammary glands (32, 35, 36). Binding to their respective receptors on the surface of phagocytes will initiate oxidative burst activity (21). A major function of mammary phagocytes is to help in the elimination of milk components and cellular debris (20), ingestion of which will also activate oxidative burst activity (6). The decrease in CL activity after exposure to zymosan for phagocytes isolated during the dry period could be partly due to previous loss of lysosomes that occurred during lysosomal fusion with phagosomes containing milk components and cellular debris (6) and due to a loss of lysosomes during migration from the circulation to the mammary gland (11). Also, the greatest decrease in CL activity occurred on d 21 when the percentage of macrophages was highest. Comparative studies with leukocytes has shown that CL activity of macrophages is lower than that for neutrophils and that macrophages require longer to reach maximal CL activity (10, 30).

## CONCLUSIONS

Results from the present investigation clearly demonstrated 1) decreased ability of secretions to support phagocytosis during the dry period, 2) inhibitory effect of secretions obtained during mammary involution on phag-

ocytosis, and 3) diminished phagocyte function with advancing stage of the dry period.

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